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Effect of extracellular calcium on regucalcin expression and cell viability in neoplastic and non-neoplastic human prostate cells

Cátia V. Vaz [1], Daniel B. Rodrigues [1], Sílvia Socorro *, Cláudio J. Maia *

CICS-UBI — Health Sciences Research Centre, University of Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal

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ABSTRACT

Extracellular calcium (Ca^{2+}_o) and its receptor, the Ca^{2+}-sensing receptor (CaSR), play an important role in prostate physiology, and it has been shown that the deregulation of Ca^{2+} homeostasis and the overexpression of CaSR are involved in prostate cancer (PCa). Regucalcin (RGN), a Ca^{2+}-binding protein that plays a relevant role in intracellular Ca^{2+} homeostasis, was identified as an under-expressed protein in human PCa. Moreover, RGN was associated with suppression of cell proliferation, suggesting that the loss of RGN may favor development and progression of PCa. This work aims to unveil the role of Ca^{2+}_o on RGN expression and viability of non-neoplastic (PNT1A) and neoplastic (LNCaP) prostate cell lines. It was demonstrated that Ca^{2+}_o up-regulates RGN expression in both cell lines, but important differences were found between cells for dose- and time-responses to Ca^{2+}_o treatment. It was also shown that high $[Ca^{2+}]_o$ triggers different effects on cell proliferation of neoplastic and non-neoplastic PCa cells, which seems to be related with RGN expression levels. This suggests the involvement of RGN in the regulation of cell proliferation in response to Ca^{2+}_o treatment. Also, the effect of Ca^{2+}_o on CaSR expression seems to be dependent of RGN expression, which is strengthened by the fact that RGN-knockdown in PNT1A cells increases the CaSR expression, whereas transgenic rats overexpressing RGN exhibit low levels of CaSR. Overall, our results highlighted the importance of RGN as a regulatory protein in Ca^{2+}-dependent signaling pathways and its deregulation of RGN expression by Ca^{2+}_o may contribute for onset and progression of PCa.

1. Introduction

Prostate cancer (PCa) is the most common male cancer concerning the number of new cases diagnosed, and represents one of the major causes of cancer death in men worldwide [1,2]. Apart from androgens, it is well known the role of calcium (Ca^{2+}) in the physiology of prostate, and the deregulation of Ca^{2+} homeostasis has been intimately associated with development and progression of PCa [3]. Over recent years, a growing body of evidence has indicated that extracellular Ca^{2+} (Ca^{2+}_o), mainly through the activity of extracellular calcium-sensing receptor (CaSR), regulates cell proliferation, differentiation, and apoptosis [4]. In addition, several studies have pointed out that the activation of CaSR is associated with the onset of PCa metastasis [5,6], which strongly highlights the importance of Ca^{2+} signaling and homeostasis in the pathophysiology of prostate gland.

Regucalcin (RGN) is a Ca^{2+}-binding protein that plays an important role in maintaining intracellular Ca^{2+} homeostasis by regulating the activity of Ca^{2+} pumps in plasma membrane and endoplasmic reticulum, and the uptake of Ca^{2+} by mitochondria [7,8]. Also, it was reported that overexpression of RGN suppresses the expression of CaSR [9]. Noteworthy RGN was described as an underexpressed protein in different types of human cancer cell lines and tissues, namely, in the hepatocellular carcinoma (HCC), and in breast and prostate cancers [10–13]. Our previous work also demonstrated that the loss of RGN expression in human PCa cases is negatively correlated with the cellular differentiation of adenocarcinoma [12]. Moreover, it was recently shown that RGN decreases cell proliferation in rat prostate through regulating the expression of cell cycle regulators [14], which supports that reduced levels of RGN may be implicated in development and progression of prostate tumors. Regarding the regulation of RGN expression, it was demonstrated that DHT down-regulates RGN levels in rat prostate and in human androgen-sensitive LNCaP PCa cells. However, the myriad of factors that maintain RGN expression levels in human prostate cells is not entirely known. Considering the importance of Ca^{2+} in prostate pathophysiology, and the role of RGN in Ca^{2+} homeostasis and cell proliferation, this work aims to determine the effect of $[Ca^{2+}]_o$ on the

Abbreviations: Ca^{2+}, Calcium; PCa, Prostate cancer; RGN, Regucalcin; HCC, hepatocelular carcinoma; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; CaSR, calcium-sensing receptor; Tg-RGN, Sprague Dawley transgenic rats overexpressing RGN; qPCR, Quantitative Real-Time PCR; PBS, phosphate buffer saline.

* Corresponding authors at: Faculdade de Ciências da Saúde, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal.
E-mail addresses: ssocorro@fcsaude.ubi.pt (S. Socorro), cmaia@fcsaude.ubi.pt (C.J. Maia).

[1] Both authors contributed equally.

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expression of RGN, and on the viability of human non-neoplastic and neoplastic prostate cells. In addition, the expression of CaSR in human non-neoplastic and neoplastic prostate cells, as well as, in the prostate of transgenic animals overexpressing RGN (Tg-RGN), was also investigated.

2. Materials and methods

2.1. Cell lines and experimental layout

The human PCa cell lines, LNCaP and PC3, and the immortalized non-neoplastic human prostate epithelial cell line, PNT1A, were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). All prostate cell lines were cultured in DMEM 21068 (Gibco, Paisley, UK), supplemented with 1.8 mM $CaCl_2$ (VWR, Leuven, Belgium), 10% FBS (Biochrom, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen) at 37 °C in an incubator equilibrated with 5% CO_2.

Cells were grown up to 60% confluence, and 24 h before stimulation, culture medium was changed to a Ca^{2+} free medium, DMEM 21068 (Gibco) containing 10% FBS (Biochrom) and 1% penicillin/streptomycin (Invitrogen). Cultured cells were maintained for additional 24 h and then exposed to different concentrations of $CaCl_2$ (0, 1, 1.8, 3, 5 and 10 mM) for 0, 1.5, 3, 6, 12, 24, 48 and/or 72 h. During the time-course experiment for different Ca^{2+} stimuli, cells were harvested and stored at −80 °C until RNA and protein extraction, or cell viability assays were performed.

For RGN gene knockdown in PNT1A and LNCaP cells, several transfection conditions were previously optimized. Cells were seeded in six plate multiwells and at 40% confluence were transfected with 10 nM of a small interfering RNA (siRNA) targeting the RGN (s17374) (Ambion, USA) and 5 μL of lipofectamine 2000 (Invitrogen, USA) for 24 h in Opti-MEM medium (Invitrogen), following manufacturer's instructions. As control for RGN specific targeting, a Scramble siRNA sequence (AM4635) (Ambion, USA) was used. The medium was replaced to DMEM 21068 (Gibco) supplemented with 1.8 mM $CaCl_2$ (VWR), 10% FBS (Biochrom) and 1% penicillin/streptomycin (Invitrogen), and the RGN gene knockdown was confirmed by Western blot after 36 h of incubation with the siRNA.

2.2. Animals

Wild-type male rats (*Rattus norvegicus*) of Sprague Dawley strain were obtained from Charles River (Barcelona, Spain). Sprague Dawley transgenic rats overexpressing RGN (Tg-RGN) were originally generated by Yamaguchi M by oocyte transgene pronuclear injection [15] and were purchased from Japan SLC (Hamamatsu, Japan) that commercializes the strain. All rats were euthanized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France) and whole prostates were removed and frozen in liquid nitrogen for protein extraction.

2.3. Total RNA extraction and cDNA synthesis

Total RNA from human prostate cell lines was extracted using the TRI reagent (Sigma-Aldrich, Saint Louis, Missouri, USA) according to the manufacturer's instructions. The quantity and quality of extracted RNA were assessed by spectrophotometry (Pharmacia Biotech, Ultrospec 3000, Cambridge, England) and agarose gel electrophoresis, respectively. For cDNA synthesis, 1 μg of total RNA was denatured for 5 min at 70 °C together with 250 ng of random hexamer primers (Invitrogen), and reverse transcription was carried out at 37 °C for 60 min in a 20 μl reaction containing reverse transcriptase buffer, 1 μl of dNTP Mix (10 mM each; GE Healthcare, Buckinghamshire, UK) and 200 U of M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). The reaction was stopped at 75 °C for 15 min and synthesized cDNA was stored at −20 °C until further use.

2.4. Quantitative Real-Time PCR (qPCR)

Quantitative Real-Time (qPCR) was carried out to evaluate the mRNA expression of RGN in human prostate cell lines (PNT1A and LNCaP), in response to Ca^{2+} stimuli. Specific primers for human RGN (Sense: 5′-GCAAGTACAGCGAGTGACC-3′; antisense: 5′-TTCCCATCAT TGAAGCGATTG-3′) amplified a fragment of 177 bp. Human beta-2-microglobulin (β_2M) (Sense: 5′-ATGAGTATGCCTGCCGTGTG-3′; antisense: 5′-CAAACCTCCATGATGCTGCTTAC-3′) and GAPDH (sense: 5′-CGCCAGCCGAGCCACATC-3′; antisense: 5′-CGCCCAATACGACCAAAT CCG-3′) primers were used as internal controls to normalize RGN expression. qPCR reactions were carried out in IQ5 system (Bio-Rad, Hercules, USA) and the efficiency of amplifications was determined for all primer sets using serial dilutions of cDNA (1, 1:10 and 1:100). PCR conditions and reagents concentrations were previously optimized and the specificity of the amplicons was determined by melting curves analysis. For qPCR reactions, 1 μl of synthesized cDNA was used in a 20 μl reaction containing 10 μl Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Fermentas, Burlington, Canada) and sense and antisense primers (200 nM for RGN and 300 nM for β_2M and GAPDH). Reaction conditions comprised 5 min denaturation at 95 °C, followed by 35 cycles of 95 °C for 10 s, a specific annealing temperature of 60 °C for 30 s and 72 °C for 10 s. Samples were run in triplicate in each PCR assay. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$ [16].

2.5. Western blot

Total proteins were extracted from human prostate cell lines (PNT1A and LNCaP) and rat prostate using RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8 and 1 mM EDTA) supplemented with protease inhibitors cocktail. Protein concentration was determined by the Bradford assay (Bio-Rad) and approximately 60 μg of cell and tissue protein extracts was resolved by SDS-PAGE on 12% gels and electrotransferred to a PVDF membrane (GE Healthcare). Membranes were incubated overnight at 4 °C with mouse anti-RGN (1:1000, ab81721, Abcam, Cambridge, United Kingdom) or mouse anti-calcium sensing receptor clone HL1499 (1:1000, C0493, Sigma-Aldrich) primary antibodies. A mouse anti-β-actin antibody (1:5000, A5441, Sigma-Aldrich) was used for normalization of protein expression. Membranes were incubated for 1 h with goat anti-mouse IgG + IgM-AP (1:5000, NIF1316; GE Healthcare) used as secondary antibody. Finally, membranes were incubated with ECF substrate (GE Healthcare) for 3 min, and visualized on the Molecular Imager FX Pro plus MultiImager (Bio-Rad). Band densities were obtained according to standard methods using the Quantity One Software (Bio-Rad) and normalized by division with the respective β-actin band density.

2.6. Immunofluorescence microscopy

PNT1A and LNCaP cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 during 5 min. A blocking step was performed by incubating cells with 20% FBS in phosphate buffer saline (PBS) containing 0.1% tween®-20 (PBST) for 1 h at room temperature, and then, cells were incubated with anti-RGN antibody (1:50, ab81721, Abcam) for 1 h at room temperature. The Alexa fluor 488 and 546 conjugated goat anti-mouse IgG (Invitrogen) were used as secondary antibodies. The specificity of the staining was accessed by omission of the primary antibody. Cell nuclei were stained with Hoechst 33342 (10 mg/ml, Invitrogen) for 10 min. Lamellae were washed and mounted onto microscope slides with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany).

2.7. Cell viability assay

PNT1A and LNCaP cells were seeded into 96-well plates at a density of 1.5×10^3 cell per well in 100 μl of cell medium culture, and exposed to 0, 1, 1.8, 3, 5 and 10 mM of $CaCl_2$ for 0, 6, 12, 24, 48 and 72 h, as described above. Cell viability was determined by a colorimetric (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2 M) tetrazolium (MTS) assay using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). The conversion of MTS compound to the colored formazan product was detected by measurement of absorbance at 490 nm in a Microplate Reader (Biochrom, Anthos 2020). The relative number of viable cells in each experimental condition was calculated by normalizing the absorbance to that of the corresponding control. All experiments were repeated at least three times, and each experiment was carried out in hexaplicated.

2.8. Statistical analysis

The statistical significance of differences in mRNA and protein expression and cell viability among experimental groups was assessed by Student's t-test or by ANOVA followed by the Bonferroni test. Significant differences were considered when $P < 0.05$ (*,#), $P < 0.01$ (**, ##) or $P < 0.001$ (***, ###). All experimental data are shown as mean ± SEM. The Graphpad Prism 5.0 program (GraphPad Software, San Diego, CA, USA) was used for this analysis.

3. Results

3.1. Regucalcin expression is significantly diminished in human prostate cancer cell lines

The mRNA and protein expression of RGN in neoplastic (LNCaP and PC3) and non-neoplastic (PNT1A) human prostate cells, was compared by means of qPCR and Western blot analysis (Fig. 1). The expression of RGN mRNA (Fig. 1A) and protein (Fig. 1B) was significantly decreased in both PCa cell lines comparatively with PNT1A cells (LNCaP cells, 0.24- and 0.79-fold variation for mRNA and protein, respectively; PC3 cells, 0.46- and 0.83-fold variation for mRNA and protein, respectively).

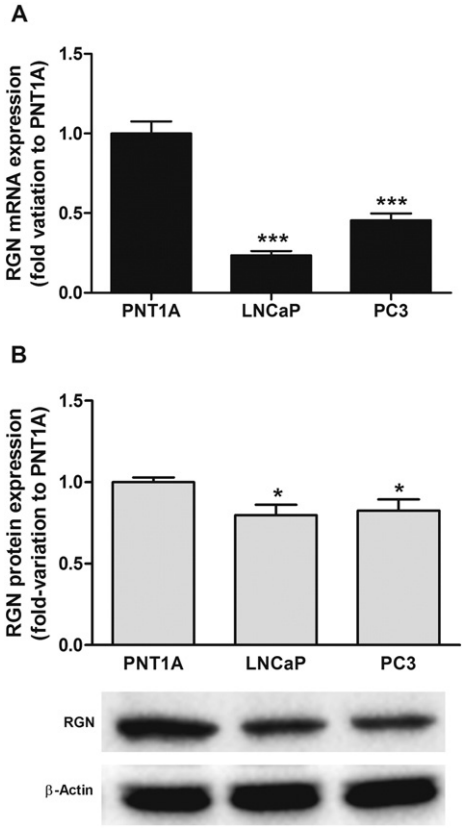

Fig. 1. RGN mRNA (A) and protein (B) expression in non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) human prostate cell lines. mRNA expression was determined by qPCR after normalization with β_2M and GAPDH housekeeping genes. Protein expression was analyzed by Western Blot and normalized with β-actin. All results are expressed as fold-variation relatively to PNT1A. Error bars indicate mean ± SEM, n = 5. * $P < 0.05$; *** $P < 0.001$. Representative immunoblots are shown in bottom panel.

3.2. Extracellular calcium exerts a biphasic effect on regucalcin expression

Considering the role of RGN in Ca^{2+} homeostasis, we have hypothesized that $Ca^{2+}{}_o$ regulates RGN gene expression, and that this effect may differ between neoplastic and non-neoplastic prostate cells. Taking into account that RGN is an androgen-responsive gene [12,14,17], we have used the androgen-sensitive LNCaP and the non-neoplastic PNT1A cell lines to evaluate the responsiveness of RGN expression to $Ca^{2+}{}_o$.

qPCR results demonstrated that $Ca^{2+}{}_o$ exerts a biphasic effect on RGN mRNA expression in both cell lines. In PNT1A cells, it was observed an up-regulation of RGN after 1.5 h of stimulation with 1, 1.8 and 3 mM $Ca^{2+}{}_o$ (respectively, 1.48-, 1.6- and 1.71-fold variation relatively to PNT1A untreated cells; Fig. 2A).

Regarding LNCaP cells, a significant up-regulation was only visible after 3 h of treatment with 1.8 mM $Ca^{2+}{}_o$ (1.7-fold variation relatively to LNCaP untreated cells; Fig. 2B). Thereafter, the levels of RGN were reduced along the experimental time-course, and a significant down-regulation of RGN mRNA was observed in both cell lines after 12 h of stimulation with $[Ca^{2+}]_o$ of 1, 1.8, 3, 5 and 10 mM (respectively, 0.40-, 0.36-, 0.39-, 0.71- and 0.53-fold variation in PNT1A cells relatively to control untreated group; and 0.68-, 0.70-, 0.64-, 0.70- and 0.59-fold variation in LNCaP cells relatively to control untreated group).

Considering 1.8 mM as the physiological $[Ca^{2+}]_o$, the effects of low (1 mM) and high (5 and 10 mM) doses of $Ca^{2+}{}_o$ were also compared to 1.8 mM $Ca^{2+}{}_o$. In PNT1A cells, the results showed a diminished expression of RGN after 1.5 h of stimulation with 5 and 10 mM (respectively, 43% and 48% of reduction in PNT1A cells relatively to 1.8 mM $Ca^{2+}{}_o$). However, a significant increase in RGN levels was observed at 3 and 12 h after treatment with 5 mM $Ca^{2+}{}_o$ (respectively, 36 and 49% of reduction in PNT1A cells relatively to 1.8 mM $Ca^{2+}{}_o$). The treatment with 10 mM $Ca^{2+}{}_o$ decreased the levels of RGN in PNT1A cells at 3 h (30%), but increased at 12 h (32%) relatively to 1.8 mM $Ca^{2+}{}_o$ (Fig. 2A).

In LNCaP cells, a significant reduction in RGN levels was observed with 3 mM $Ca^{2+}{}_o$ at 3 and 6 h (respectively, 41 and 51% reduction relatively to 1.8 mM $Ca^{2+}{}_o$). Also, a significant reduction in RGN expression was observed after treatment of LNCaP cells with 5 and 10 mM $Ca^{2+}{}_o$ (respectively, 39 and 50% at 1.5 h; 49 and 69% at 3 h; and 62 and 50% at 6 h relatively to 1.8 mM $Ca^{2+}{}_o$). At 12 h of stimulation, no significant differences were perceived for the effect of 1, 3, 5 and 10 mM $Ca^{2+}{}_o$ comparatively with the 1.8 mM $Ca^{2+}{}_o$. These results suggest a time and dose-dependent effect of $Ca^{2+}{}_o$ on RGN expression in human prostate cells. Subsequently, the RGN protein expression in response to Ca^{2+}-stimulation was investigated by means of Western blot and immunofluorescence analysis. $Ca^{2+}{}_o$ treatment for 1.5 h and 3 h was the experimental time-points selected for protein analysis, respectively, in PNT1A and LNCaP cells, as it corresponded to the increased expression of RGN mRNA relatively to untreated control group. Dose-response analysis for different $[Ca^{2+}]_o$ demonstrated that PNT1A cells treated with 1, 1.8 and 3 mM $Ca^{2+}{}_o$ for 1.5 h show increased expression of RGN protein (respectively, 2.11-, 1.96- and 1.59-fold variation relatively to PNT1A untreated cells; Fig. 2C), whereas no changes were observed in the experimental group treated with high $[Ca^{2+}]_o$.

In accordance with the results of mRNA expression, a significant reduction on RGN protein levels after treatment with 5 and 10 mM

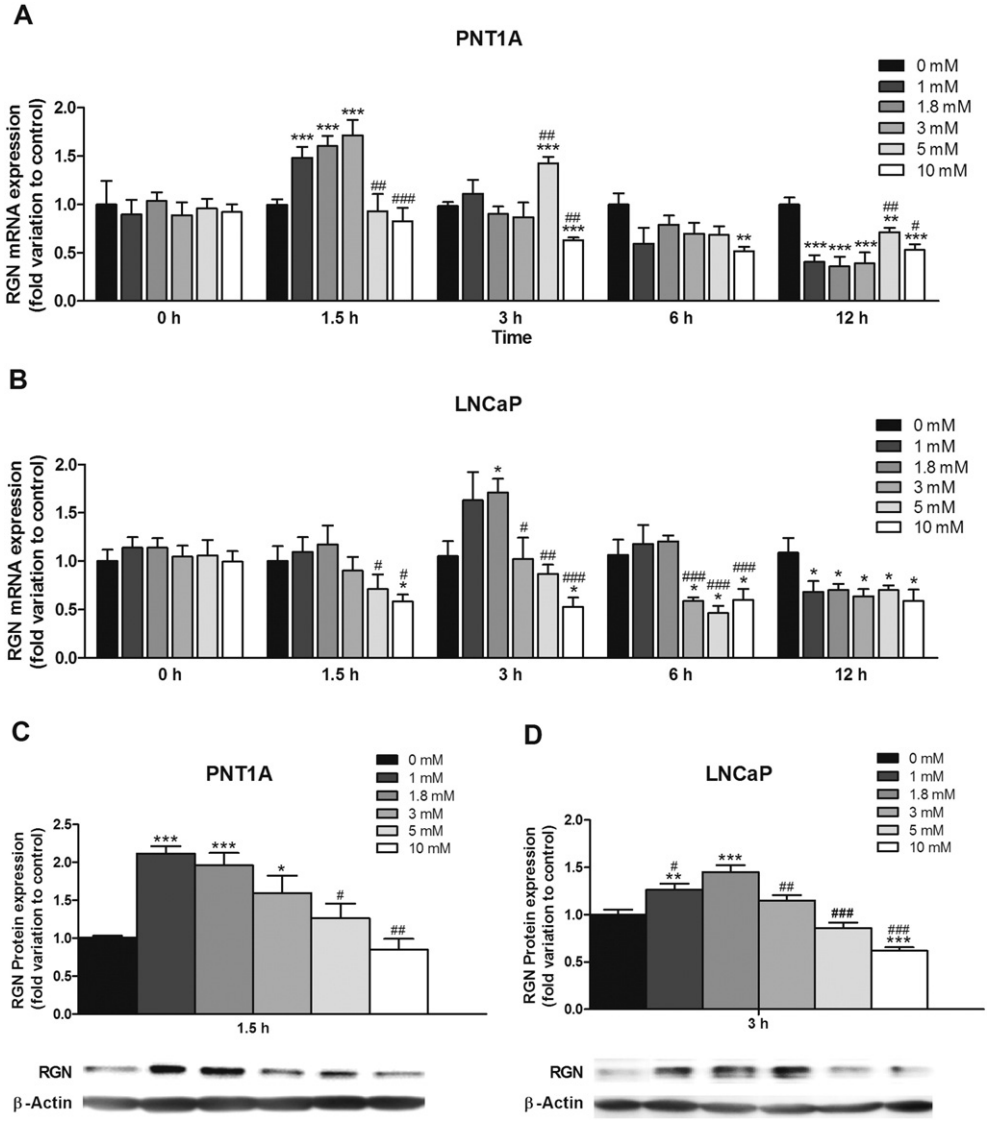

Fig. 2. Effect of extracellular Ca^{2+} (Ca^{2+}_o) on RGN expression in PNT1A and LNCaP cells. mRNA expression of RGN (A, B) was determined by qPCR after normalization with β_2M and GAPDH housekeeping genes. RGN protein expression (C, D) was analyzed by Western blot and normalized with β-actin. All results are expressed as fold-variation relatively to the control untreated group (0 mM Ca^{2+}_o). Error bars indicate mean ± SEM, n = 6. *, # $P < 0.05$; **, ## $P < 0.01$; ***, ### $P < 0.001$ relatively to untreated cells or 1.8 mM Ca^{2+}_o, respectively. Representative immunoblots are shown in bottom panel.

Ca^{2+}_o (respectively, 36 and 57% in PNT1A cells relatively to 1.8 mM Ca^{2+}_o, Fig. 2C) was also observed.

Also, LNCaP cells treated with 1 and 1.8 mM of Ca^{2+}_o for 3 h, displayed increased RGN protein expression (respectively, 1.26- and 1.45-fold variation relatively to LNCaP untreated cells; Fig. 2D), whereas the expression of RGN is diminished in cells stimulated with 10 mM of Ca^{2+}_o (0.62-fold variation relatively to LNCaP untreated cells; Fig. 2D). Comparing the effect of high doses (3, 5 and 10 mM Ca^{2+}_o) with the physiological dose, a significantly decreased protein expression of RGN was observed in LNCaP cells (respectively, 21, 41 and 57% of reduction relatively to 1.8 mM Ca^{2+}_o, Fig. 2D).

Comparing the effect of Ca^{2+}_o between non-neoplastic and neoplastic human prostate cell lines, it is possible to infer that Ca^{2+}_o-induced up-regulation of RGN protein expression is more pronounced in PNT1A cells than in LNCaP cells. Immunofluorescence results (Fig. 3) also showed enhanced staining of RGN in the cytoplasm of PNT1A and LNCaP cells treated with 1.8 mM Ca^{2+}_o for 1.5 h and 3 h, respectively. This enhancement also was evident in the nucleus of PNT1A cells.

3.3. Cell viability of prostate cells is dependent on extracellular calcium concentration

Considering that Ca^{2+}_o can affect cell growth rate, we evaluated the effect of several $[Ca^{2+}]_o$ on the viability of PNT1A (Fig. 4A) and LNCaP (Fig. 4B) cells for 72 h by means of MTS assay. PNT1A cells stimulated with 3, 5 and 10 mM of Ca^{2+}_o displayed diminished cell viability comparatively with the control untreated group (**$P < 0.01$ or ***$P < 0.001$) throughout the experimental time-course. For 48 h of stimulation with 1.8 mM Ca^{2+}_o, the percentage of viable PNT1A cells was enhanced when compared to control group, increasing even further thereafter. On the other hand, the viability of LNCaP cells after 48 h of treatment was stimulated with 1.8 mM or higher $[Ca^{2+}]_o$ relatively to untreated cells or cells treated with 1 mM of Ca^{2+}_o (***$P < 0.001$).

Comparing the effect of high and low $[Ca^{2+}_o]$ on cell viability, it was observed a diminished viability in both cell lines when compared to the physiological dose of Ca^{2+}_o (1.8 mM, Fig. 4).

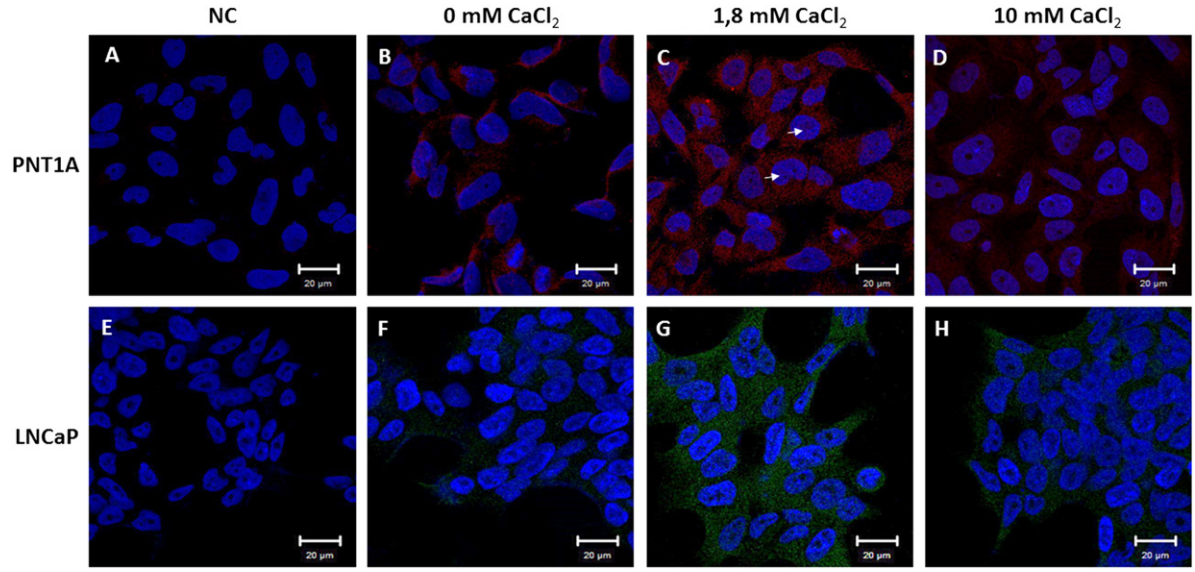

Fig. 3. Representative confocal microscopy images showing the expression and subcellular localization of RGN in PNT1A (red) and LNCaP (green) cells treated with 0, 1.8 and 10 mM of $Ca^{2+}{}_o$ for 1.5 h and 3 h, respectively. Nuclei are stained with Hoechst 33342 (blue). Negative controls (NC) for RGN were obtained by omission of the primary antibody. Co-localization of RGN and nucleus is shown by the inserted arrows.

3.4. Extracellular calcium regulates the expression of calcium-sensing receptor

Even small changes in $[Ca^{2+}]_o$ are sensed by the G-protein coupled receptor CaSR, which upon ligand binding translates this information to intracellular signaling pathways [4]. So, we first analyzed the expression of CaSR protein in PNT1A and LNCaP cells by means of Western blot analysis. The obtained results demonstrated that LNCaP cells express higher levels of CaSR relatively to the non-neoplastic PNT1A cells (2.08-fold variation; Fig. 5A). Considering the effect of $[Ca^{2+}]_o$ on the expression of RGN and viability of PNT1A and LNCaP cells, together with other reports indicating that up-regulation of RGN expression may influence the expression of CaSR [9], we decided to evaluate the effect of $Ca^{2+}{}_o$ on the expression of CaSR in these human prostate cell lines. The protein levels of CaSR in response to 1.8 mM $Ca^{2+}{}_o$ in PNT1A and LNCaP cells were determined by Western blot analysis. The results obtained showed that CaSR protein expression significantly decreased in PNT1A cells treated with 1.8 mM of $Ca^{2+}{}_o$ for 3 h and 6 h when compared with the control group (0.7-fold variation relatively to PNT1A untreated cells; Fig. 5B). In what concerns LNCaP cells, no significant differences were observed on CaSR protein expression between the experimental groups (Fig. 5C).

3.5. RGN down-regulates the expression of CaSR

In order to investigate the role of RGN on the regulation of CaSR, we analyzed the expression of CaSR protein in PNT1A and LNCaP cells after RGN knockdown. In addition, the expression of CaSR was compared between the prostate of Tg-RGN animals and their wild-type counterparts. As expected, the levels of RGN in PNT1A and LNCaP cells decreased after transfection with siRNA targeting RGN (respectively, 0.27 and 0.56-fold variation relatively to control cells, $^{*}P < 0.05$; Fig. 5D and E). Western blot results demonstrated that RGN knockdown induces the expression of CaSR in PNT1A cells (1.21-fold variation relatively to control cells, $^{*}P < 0.05$; Fig. 5D), but no differences were observed in LNCaP cells (Fig. 5E). Accordingly, transgenic overexpression of RGN down-regulates CaSR levels. The prostates of Tg-RGN rats displayed diminished expression of CaSR protein relatively to the

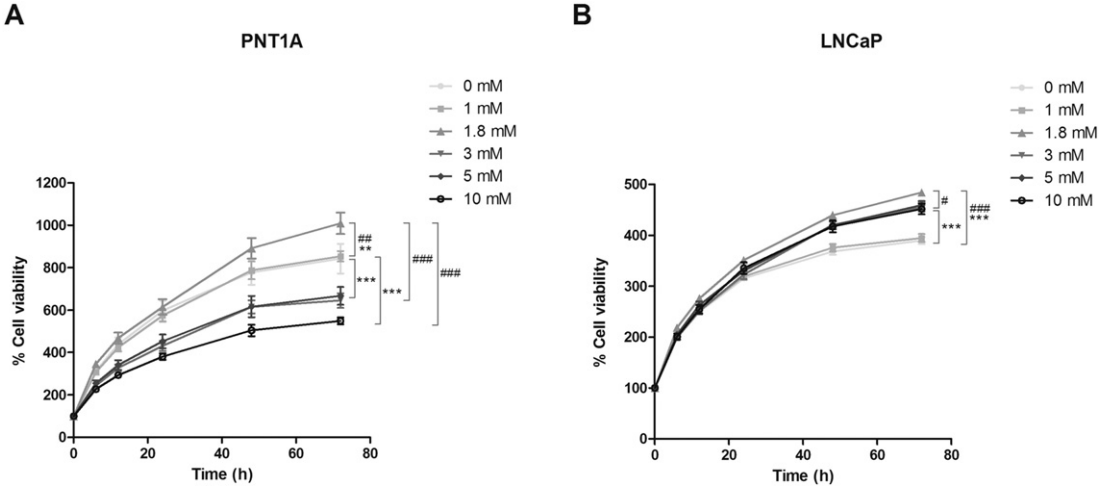

Fig. 4. Effect of extracellular Ca^{2+} on the viability of PNT1A and LNCaP cells determined by the MTS assay. Error bars indicate mean ± SEM, n = 6. $^{\#}P < 0.05$ relatively to 1.8 mM $Ca^{2+}{}_o$; **, $^{\#\#}P < 0.01$ and ***, $^{\#\#\#}P < 0.001$ relatively to untreated cells or 1.8 mM $Ca^{2+}{}_o$, respectively.

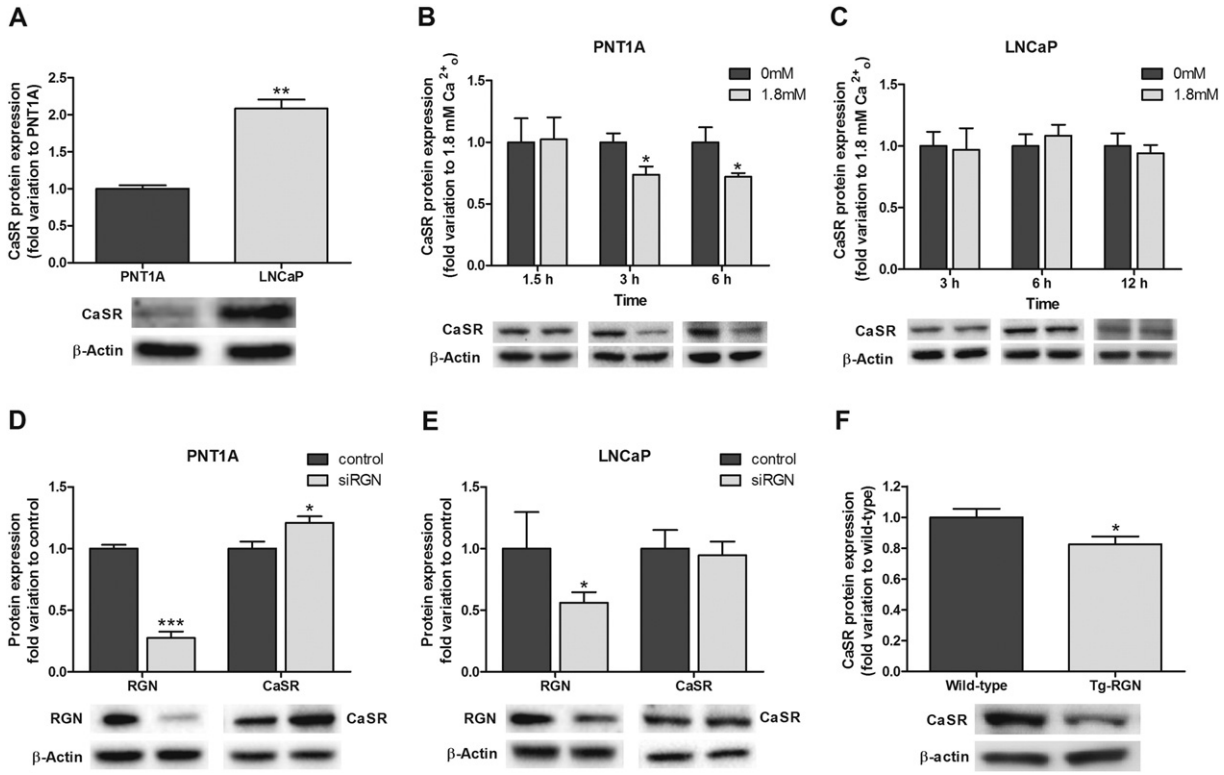

Fig. 5. CaSR protein expression in human prostate cell lines (PNT1A and LNCaP) (A), and effect of 1.8 mM of extracellular Ca^{2+} on CaSR expression in PNT1A (B) and LNCaP (C) cells. RGN gene knockdown and its effect on CaSR expression in PNT1A (D) and LNCaP (E) cells, and CaSR protein expression in the prostate of transgenic rat overexpressing RGN (Tg RGN) comparatively with their wild type counterparts (F). Results are expressed as fold-variation relatively to the control groups. Protein expression was analyzed by Western blot and normalized with β-actin. Representative immunoblots are shown. Error bars indicate mean ± SEM, n = 6. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

wild-type group (0.82-fold variation, *$P < 0.05$; Fig. 5F). Taken together, these results indicate that RGN may control the expression levels of CaSR in prostate cells.

4. Discussion

The onset and progression of PCa are intimately dependent on the regulation of different signaling pathways controlled by androgens and Ca^{2+} [3,18], which also includes the influence of $Ca^{2+}{}_o$ through the activity of CaSR. Although the mechanisms underlying Ca^{2+} actions in PCa are not completely elucidated, several studies have linked the CaSR, and also the RGN protein, with prostate carcinogenesis.

It has been demonstrated that loss of RGN expression is associated with progression of PCa [12] and that RGN attenuates proliferation of prostate cells [14], suggesting that this Ca^{2+}-binding protein may have a protective role against the development of prostate tumors. In this way, it is absolutely crucial to characterize the factors that control RGN expression in prostate cells under normal and pathological conditions. This work first intended to ascertain the effect of $Ca^{2+}{}_o$ on the expression of RGN in neoplastic and non-neoplastic prostate cell lines. As a starting point, the expression of RGN was analyzed in non-neoplastic, PNT1A cells, and in androgen-responsive and androgen non-responsive neoplastic cell lines, LNCaP and PC3, respectively. LNCaP and PC3 cells displayed lower levels of RGN in comparison with PNT1A cells, which is in agreement with other studies demonstrating that RGN is underexpressed in human PCa cases [12]. Also, the reduced levels of RGN in LNCaP cells are concordant with the previously described down-regulatory effect of DHT on RGN expression in PCa cells [12]. Nevertheless, other factors must be involved in the diminution of RGN levels in PCa, since androgen-insensitive PC3 cells also displayed reduced expression of RGN. Taking into account the present findings, and our previous data indicating RGN as an androgen-responsive gene [14,19,20], PNT1A and LNCaP cells were selected to investigate the role of $Ca^{2+}{}_o$ in regulating the expression of RGN. The results obtained demonstrated that $Ca^{2+}{}_o$ induced time- and dose-dependent effects on the expression of RGN. A physiological $[Ca^{2+}]_o$ (1.8 mM) promptly increased RGN mRNA and protein levels in PNT1A and LNCaP cells for 1.5 h and 3 h of stimulus, respectively. However, when cells were stimulated with higher $[Ca^{2+}]_o$ (3 mM and 5 mM) the effect on up-regulation of RGN expression was only observed in PNT1A cells, which indicates that the regulation of RGN expression by $Ca^{2+}{}_o$ differs between non-neoplastic and neoplastic prostate cells. Others have described the regulation of RGN by $Ca^{2+}{}_o$ in rat and mice liver, with RGN mRNA being markedly increased at 30 and 60 min after intraperitoneal administration of $CaCl_2$ [21,22], or between 30 and 180 min following a single oral administration of $CaCl_2$ solution [23]. The stimulatory effect on RGN concentration was also described in the rat kidney cortex 60 to 120 min after of a single intraperitoneal administration of a $CaCl_2$ solution [24].

Interestingly, when PNT1A and LNCaP cells were treated for longer periods (6 to 12 h), the levels of RGN mRNA and protein were down-regulated relatively to untreated cells, suggesting that RGN may trigger a negative feedback in order to decrease its cell levels and avoid an over-stimulation by RGN. Considering that RGN has been involved in the regulation of Ca^{2+}-dependent intracellular signaling pathways [25], and the importance of $Ca^{2+}{}_o$ in prostate cells, it is liable to speculate that the up-regulation of RGN expression in response to $Ca^{2+}{}_o$ may play an important role in prostate physiology. In addition, it also should be highlighted the $Ca^{2+}{}_o$ effects relatively to 1.8 mM $Ca^{2+}{}_o$, which is considered the physiological $[Ca^{2+}]_o$. The present results showed that extreme concentrations of $Ca^{2+}{}_o$, namely high levels, decrease the expression of RGN in both cell lines relatively to physiological $[Ca^{2+}]_o$. Therefore, it is liable to speculate that changes in serum $[Ca^{2+}]_o$ may decrease the expression of RGN, and consequently, favor the proliferation of prostate cells and tumor development and progression. Indeed, although there are contradictory results, some epidemiologic

studies have found that higher levels of Ca^{2+}_o are associated with PCa risk [26,27].

Our research group previously demonstrated that prostates from Tg-RGN rats present reduced cell proliferation underpinned by decreased expression of the oncogene H-Ras and increased levels of the cell cycle inhibitor p21 [14]. Although it has not been demonstrated the role of RGN as a transcription regulator, the immunofluorescence results obtained herein showed increased accumulation of RGN in the nucleus, namely in PNT1A cells, suggesting that RGN may play a role in regulating the nuclear function. These results are corroborated by other studies, which also demonstrated the presence of RGN in the nucleus of several human cell types including prostate cells, and suggested that it may regulate gene expression [9,12,28].

Considering the available reports describing the effect of Ca^{2+}_o on cell proliferation [4], the present study also evaluated how distinct $[Ca^{2+}]_o$ influence the proliferation of PNT1A and LNCaP cells. Remarkably, the stimulation with high $[Ca^{2+}]_o$ (3, 5 and 10 mM) triggered different responses in non-neoplastic and neoplastic cell lines. In PNT1A cells, high $[Ca^{2+}]_o$ decreased cell viability comparatively with the control but the opposite effect was observed in LNCaP cells. Noteworthy, the perceived effects on cell proliferation followed concomitant responses on the expression of RGN, since it was always visible, except with 10 mM Ca^{2+}_o, an up-regulation of RGN expression in PNT1A cells, whereas in LNCaP cells this effect was only visible with physiological dose. Our results also showed that both cell lines presented decreased proliferation when treated with low or high concentrations of Ca^{2+}_o relatively to 1.8 mM Ca^{2+}_o. However, the diminished proliferation in response to high doses of Ca^{2+}_o is less evident in LNCaP cells, suggesting that cancer cells may become more sensitive to proliferation than normal cells in the presence of high concentrations of Ca^{2+}_o. Altogether, and even considering many other mechanisms involved in the action of Ca^{2+}_o, our results suggest that the up-regulation of RGN in response to physiological $[Ca^{2+}]_o$ may play an important role in protecting cells from proliferation. These observations are supported by previews findings demonstrating that overexpression of RGN suppresses cell proliferation in NRK52E and hepatoma H4-II-E cells, and in rat prostate by decreasing the expression of cell cycle regulators [14,29–32]. Nevertheless, other studies are required to fully clarify the impact of RGN levels on the regulation of cell proliferation in neoplastic and non-neoplastic prostate cell lines.

It is well described that one of the mechanisms of Ca^{2+}_o action is through its cognate receptor, the CaSR [4]. It has been reported that Ca^{2+} and the CaSR are important players in tumorigenesis, and in PCa, the CaSR is even over-expressed [6]. Therefore, we compared the expression of CaSR between PNT1A and LNCaP cells. Our results showed that LNCaP cells display higher levels of CaSR than PNT1A cells, which is in accordance with previous findings describing a positive correlation between activation of CaSR and development of PCa [6]. We investigated the putative role of RGN controlling CaSR expression in PNT1A and LNCaP cells after treatment with 1.8 mM Ca^{2+}_o. Interestingly, a decreased expression of CaSR protein was only observed in PNT1A cells after 3 h of treatment, but no significant changes were found in LNCaP cells up to 12 h of treatment. Considering the high levels of RGN at 1.5 h after treatment with physiological $[Ca^{2+}]_o$ in PNT1A cells, these results suggest that the down-regulation of CaSR may occur due to the higher levels of RGN induced by this $[Ca^{2+}]_o$. Inversely, the reduced levels of RGN in LNCaP cells associated with their lower capacity to increase RGN expression in response to $[Ca^{2+}]_o$ may explain the absence of effects on the expression of CaSR in LNCaP cells treated with 1.8 mM Ca^{2+}_o. In an effort to support the hypothesis that RGN may be involved in the regulation of CaSR in prostate cells, we analyzed the expression of CaSR protein in PNT1A and LNCaP cells after RGN knockdown, and, also, in the prostate of Tg-RGN animals. The RGN gene knockdown induced the CaSR expression in PNT1A but not in LNCaP cells, suggesting that the normal role of RGN in down-regulating CaSR may be lost in cancer cells. The role of RGN in regulation of CaSR levels in normal prostate is strengthened by the fact that Tg-RGN animals displayed lower levels of CaSR in comparison with their wild type counterparts. These results are in accordance with the previously described in kidney cells, where RGN had a suppressive effect on the expression of proteins involved in intracellular Ca^{2+}-signaling pathways, including the CaSR [9]. Taken together, our results suggest that RGN may not only have an important role in the control of cell proliferation but also in desensitizing the signaling pathways triggered by CaSR. This is strongly supported by the histopathological findings in human PCa samples, which showed over-expression of CaSR and loss of RGN [12,33].

In sum, it was first demonstrated that RGN expression is regulated by Ca^{2+}_o, in non-neoplastic and neoplastic PCa cells. However, important differences on dose- and time-responses to $[Ca^{2+}]_o$ were observed between PNT1A and LNCaP cells, with PNT1A cells displaying more pronounced effects in the up-regulation of RGN by Ca^{2+}_o. In addition, a physiological $[Ca^{2+}]_o$ down-regulated CaSR expression in non-neoplastic cells, an effect not observed in PCa cells, which plausibly could be linked to the differential response in RGN expression upon treatment Ca^{2+}_o. These results strengthen the role of RGN in the modulation of intracellular Ca^{2+}-signaling pathways, and indicate that abnormalities in the $[Ca^{2+}]_o$ may deregulate RGN expression levels contributing for PCa onset and progression.

Conflict of interest

The authors declare that there is no conflict of interests.

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